Title:	Retrograde Signalling as an Informant of Circadian Timing
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Summary

The circadian system comprises of interlocking transcriptional/translational feedback loops that regulate gene expression and consequently modulate plant development and physiology. In order to maximize utility, the circadian system is entrained by changes in temperature and light, allowing endogenous rhythms to be synchronized with both daily and seasonal environmental change. While a great deal of environmental information is decoded by a suite of photoreceptors, it is also becoming apparent that changes in cellular metabolism also contribute to circadian timing, either through the stimulation of metabolic pathways or through the accumulation of metabolic intermediates as a consequence of environmental stress. As the source of many of these metabolic byproducts, mitochondria and chloroplasts have begun to be viewed as environmental sensors, and rapid advancement of this field is revealing the complex web of signalling pathways initiated by organelle perturbation. This review will highlight recent advances in our understanding of how this metabolic regulation influences circadian timing.

Keywords

Chloroplast

Circadian

MEcPP

PAP

Reactive Oxygen Species (ROS)

Retrograde

I) The circadian system is responsive to environmental change

Circadian timing modulates many biological processes, ranging from gene expression through to developmental decisions such as the transition to flowering (Millar, 2016). Although the circadian system is able to oscillate in the absence of environmental cues these endogenous rhythms are only useful if they are synchronized with and compensated against daily and seasonal changes in daylength and temperature (Millar, 2016). Plants perceive changes in their environment through the actions of photoreceptors and indicators of temperature, although this distinction is somewhat muddled as temperature and light signalling pathways share common components (Casal & Qüesta, 2018). Changes in temperature and light intensity (or quality) are sufficient to alter the expression of clock components, with the sensitivity of the circadian system to such environmental changes varying over the course of the day (Millar, 2016). This circadian 'gating' of responses to environmental change prevents the continuous resetting of the circadian timer to environmental inputs, while also allowing for the entrainment of the circadian clock to seasonal variation in daylength. Circadian gating also permits the modulation of plants' responses to environmental change (for example by enabling greater response to chilling stress during the evening).

The nuclear circadian system comprises interlocked transcriptional/translational feedback loops that combine to generate oscillations of approximately 24 hrs (Millar, 2016). Successive waves of transcriptional activators and repressors regulate gene expression, with the Myb-like transcription factors CIRCADIAN CLOCK ASSOCIATED1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY) repressing expression at dawn whereas PSEUDORESPONSE REGULATOR9 (PRR9) and its orthologues PRR7/5/TOC1 limit expression during the day (Hsu & Harmer, 2014). Later in the evening, LUX ARRHYTHMO (LUX), EARLY FLOWERING3 (ELF3), and ELF4 associate to form an Evening Complex that similarly represses gene expression (Hsu & Harmer, 2014). Transcriptional activators including REVEILLE8 (RVE8), NIGHT LIGHT-INDUCIBLE (LNK) and LIGHT-REGULATED WD (LWD) proteins complement the activity of these transcriptional repressors (Hsu & Harmer, 2014). The combined activity of these feedback loops increases the amplitude of circadian rhythms and allows the clock to be compensated against seasonal changes in temperature, as well as improving the robustness of circadian rhythmicity (Shalit-Kaneh *et al.*, 2018).

While the transcriptional components of the circadian system are comparatively well understood, how these interconnecting feedback loops are influenced by environmental

84 change remains to be fully elucidated (Hsu & Harmer, 2014). The expression of dawn-phased
85 clock components tends to be induced by light, while the Evening Complex has been
86 identified as a likely hub for signal integration as it directly interacts with the photo- and
87 thermo-sensor phytochromeB (phyB; (Hsu & Harmer, 2014; Huang, H *et al.*, 2016; Ezer *et*
88 *al.*, 2017). The role of phytochrome, cryptochrome, and UVR8 photoreceptors in the
89 circadian system has recently been discussed elsewhere (Oakenfull & Davis, 2017), and so
90 this review will instead focus upon our understanding of how metabolic changes induced by
91 environmental fluctuations affect circadian timing.
92
93 **II) Photoassimilates regulate circadian timing**
94 Plants' photosynthetic nature ensures that their metabolism is profoundly affected by the
95 availability of light, with distinct changes in cellular energy levels and photoassimilates
96 observed in the absence of this resource. Although the action of photoreceptors is sufficient
97 to entrain and maintain circadian rhythms (Millar, 2016) the metabolic consequences of
98 photosynthesis also contribute to circadian rhythms. Recent work has documented how the
99 accumulation of sucrose (and other photoassimilates) is sufficient to reset the circadian
100 system (Dalchau *et al.*, 2011; Haydon *et al.*, 2013; Haydon *et al.*, 2017). Products from
101 photosynthesis are integrated with the circadian oscillator via at least two pathways, with one
102 acting to alter phase in a PRR7- and CCA1-dependent manner (Haydon *et al.*, 2013), whereas
103 GIGANTEA promotes circadian rhythmicity in constant darkness in the presence of sucrose
104 (Dalchau *et al.*, 2011; Haydon *et al.*, 2017).
105 One possible mechanism linking photoassimilates with the circadian system revolves
106 around the SnRK1 signalling hub (Fig. 1). SnRK1 regulates metabolism by phosphorylating
107 metabolic enzymes, with its activity regulated in part through control of the AKIN10
108 catalytic subunit (Wurzinger *et al.*, 2018). SnRK1 activity is additionally repressed by the
109 accumulation of trehalose-6-phosphate, which serves as a molecular indicator of intracellular
110 sucrose (Figueroa & Lunn, 2016). Over-expression of AKIN10 extends circadian period in
111 the light (Shin *et al.*, 2017; Frank *et al.*, 2018), suggesting that control of this subunit is
112 sufficient to change circadian timing. Importantly, SnRK1 regulates the activity of bZIP63, a
113 transcription factor that activates *PRR7* transcription (Mair *et al.*, 2015; Frank *et al.*, 2018).
114 As a consequence, regulation of SnRK1 activity by trehalose-6-phospate provides a
115 mechanism by which intracellular sugar levels can be interpreted by the circadian system
116 (Frank *et al.*, 2018).
117

III) Retrograde signals contribute to circadian timing

Cell function is ultimately a cooperation between distinct organelles, which is maintained by both anterograde and retrograde signalling pathways (recently comprehensively reviewed by (Chan *et al.*, 2016b; de Souza *et al.*, 2017). Retrograde signals convey information provided by organelles such as chloroplasts and mitochondria to the nucleus, ultimately altering nuclear gene expression. For example, mature chloroplasts contribute to lipid, starch, sulfur, and amino acid metabolism, as well as contributing to the production of several hormones, in addition to their primary role in photosynthesis (Chan *et al.*, 2016b; de Souza *et al.*, 2017). Similarly, mitochondria are essential for cellular respiration and contribute to the generation of reactive oxygen species such as $^1O_2$ and H_2O_2 (Wang *et al.*, 2018). Retrograde signals consequently relay the metabolic health of the cell to the nucleus, and so provide information useful for integration into the circadian oscillator in addition to the signalling provided by photo- and thermo-sensors.

During chloroplast biogenesis, several pathways relay the general health and developmental status of the plastid to the nucleus, resulting in changes in nuclear gene expression as a consequence of biogenic control (Chan *et al.*, 2016b; de Souza *et al.*, 2017). Disruption of chloroplast function is sufficient to alter photosynthesis-associated nuclear genes (PhANGs) and nuclear circadian gene expression. For instance, loss of CHLOROPLAST RNA BINDING (CRB) delays phase and increases the amplitude of circadian gene expression (Hassidim *et al.*, 2007), while application of norflurazon (an inhibitor of photosynthesis) and lincomycin (which inhibits plastid protein synthesis) extends circadian period (Chen *et al.*, 2013). Similarly, an iron deficiency extends circadian period likely due to gross disruption of chloroplast function (Chen *et al.*, 2013; Salomé *et al.*, 2013). It is therefore apparent that retrograde signals relaying the health of the chloroplast are sufficient to delay circadian timing.

In addition to signals relaying the developmental status of organelles, the operation of mature chloroplasts can be perturbed by environmental factors (Fig. 1). Increases in irradiance promote photosynthesis and induce nonphotochemical quenching whereas changing temperatures govern the speed of enzymatic reactions. Such environmental stressors are often sufficient to induce metabolic imbalances, causing oxidative stress in mitochondria and chloroplasts and leading to changes to the energy status of the cell (Chan *et al.*, 2016b). Such disruption of metabolism additionally perturbs hormone biosynthesis and signalling, alters calcium signalling, and induces the accumulation of metabolic intermediaries that can subsequently be transported from the mitochondria or chloroplasts to

the nucleus (Chan *et al.*, 2016b; Mullineaux *et al.*, 2018). With regards to circadian rhythmicity, high temperatures are sufficient to slow the circadian oscillator, while the circadian system is also delayed by osmotic stress (Gil *et al.*, 2017; Litthauer *et al.*, 2018). As with many stress responses, the question now facing the field is whether the clock is slowed under these environmental conditions as part of a generalized reaction to cellular damage, or whether specific retrograde signals delay circadian progression as part of an adaptive response.

Reactive Oxygen Species

Reactive oxygen species (ROS) arise from the metabolic processes of mitochondria and chloroplasts (Murchie & Niyogi, 2011; Huang, S *et al.*, 2016). Both H_2O_2 accumulation and the oxidation state of peroxiredoxins (a ubiquitous family of enzymes that serve as antioxidants and regulatory proteins) vary with a circadian rhythm, suggesting that ROS generation is influenced by the circadian system (Edgar *et al.*, 2012; Lai *et al.*, 2012). Although ROS are produced as a consequence of the normal functioning of photosynthesis and respiration their accumulation increases during sub-optimal conditions, when environmental stresses such as high light induce an imbalance between photosynthetic and respiratory pathways (Mullineaux *et al.*, 2018). Under these stressful conditions, an increase in ROS production causes significant damage to the metabolic machinery, inducing photoinhibition and a generalized damage response (Mullineaux *et al.*, 2018). ROS such as H_2O_2 can be transferred directly to the nucleus from the chloroplast (Exposito-Rodriguez *et al.*, 2017), where it is likely that the activity of redox-sensitive transcription factors is modulated. However, it remains unknown how these factors contribute to circadian timing. By contrast, shorter lived ROS such as $^1O_2$ are likely to initiate signalling pathways by oxidizing carotenoids or polyunsaturated fatty acids (Mullineaux *et al.*, 2018). One such signalling trigger is β-Cyclocitral, which is an oxidised derivative of β-carotene that accumulates as a consequence of $^1O_2$ production and which induces gene expression in response to high light (Ramel *et al.*, 2012).

In addition to the direct role of ROS, or oxidized by-products, plants also have a suite of mechanisms, including non-photochemical quenching, that allow for the sequestration of ROS below critical thresholds (Ruban, 2016). Above these thresholds, ROS accumulation leads to the production of a range of metabolites that plants subsequently utilize as signalling molecules to report the metabolic status of the mitochondria and chloroplast. Such signals could be integrated into the nuclear circadian oscillator and include molecules such as

methylerythritol cyclodiphosphate (MEcPP), and 5'-Phosphoadenosine 3'-phosphate (PAP). Although these metabolites are now becoming accepted as authentic retrograde signals, it is not yet clear how these molecules integrate with circadian timing (Fig. 1).

MEcPP

Methylerythritol cyclodiphosphate (MEcPP) accumulates in response to wounding or high light stress, inducing the expression of nuclear stress-responsive genes and repressing the accumulation of auxin (Xiao *et al.*, 2012; Jiang *et al.*, 2018). Our understanding of MEcPP is still developing, but one exemplar signalling pathway repressed by MEcPP accumulation involves the transcription factor BBX19. *BBX19* transcription is repressed in *ceh1* mutants that constitutively accumulate MEcPP as a consequence of disruption of the methylerythritol phosphate pathway within the plastid (Xiao *et al.*, 2012; Wang *et al.*, 2014). BBX19 has an important role in photomorphogenesis, repressing *PIF4* and *PIF5* expression by promoting the turnover of ELF3 (Wang *et al.*, 2015). As a consequence, MEcPP accumulation results in the accumulation of ELF3. Since ELF3-overexpressing lines have previously been reported as having a long circadian period (Covington *et al.*, 2001) it is plausible that MEcPP accumulation could result in extended circadian rhythms, although this hypothesis has yet to be tested (Fig. 1).

PAP

3'-PhosphoAdenosine 5'-Phosphate (PAP) is a by-product of sulfur metabolism that is usually metabolised by SAL1, a redox-sensitive phosphatase that accumulates in both chloroplasts and mitochondria (Estavillo *et al.*, 2011; Chan *et al.*, 2016a). Following the application of high light or drought stress, SAL1 becomes oxidized, leading to its inactivation and the consequential accumulation of PAP (Estavillo *et al.*, 2011; Chan *et al.*, 2016a). PAP accumulation has several metabolic consequences, including the inactivation of XRN ribonucleases (Dichtl *et al.*, 1997; Mechold *et al.*, 2006; Fig. 1). Loss of XRN function leads to the accumulation of uncapped RNAs such as those generated from miRNA processing (Kurihara *et al.*, 2012). Interestingly, either the accumulation of PAP or the loss of XRN activity is sufficient to extend circadian period (Litthauer *et al.*, 2018). Although the mechanism underlying this phenotype remains unclear, such data are consistent with previous reports that gross defects in RNA processing slow circadian progression (Nolte & Staiger, 2015). It will therefore be important to examine how loss of XRN activity alters the stability

of circadian transcripts, and to understand how such post-transcriptional regulation acts in concert with previously reported variations in splicing to control functional gene expression.

IV) Conclusions

Plants are highly sensitive to environmental change, and we have identified a complex suite of photo- and thermo-sensors that enable these responses, as well as the circadian system that anticipates and integrates these signals to regulate development and plants responses. However, it is also important to note the contribution of metabolism-induced signalling pathways to plants responses. We are beginning to appreciate how photosynthesis and metabolic perturbations within organelles lead to differential nuclear gene expression, and consequently to circadian timing. Understanding how mitochondrial and chloroplastic signalling contribute to the circadian system (and how these pathways are reciprocally modulated by the circadian system) will allow the development of a holistic understanding of plants' responses to environmental change.

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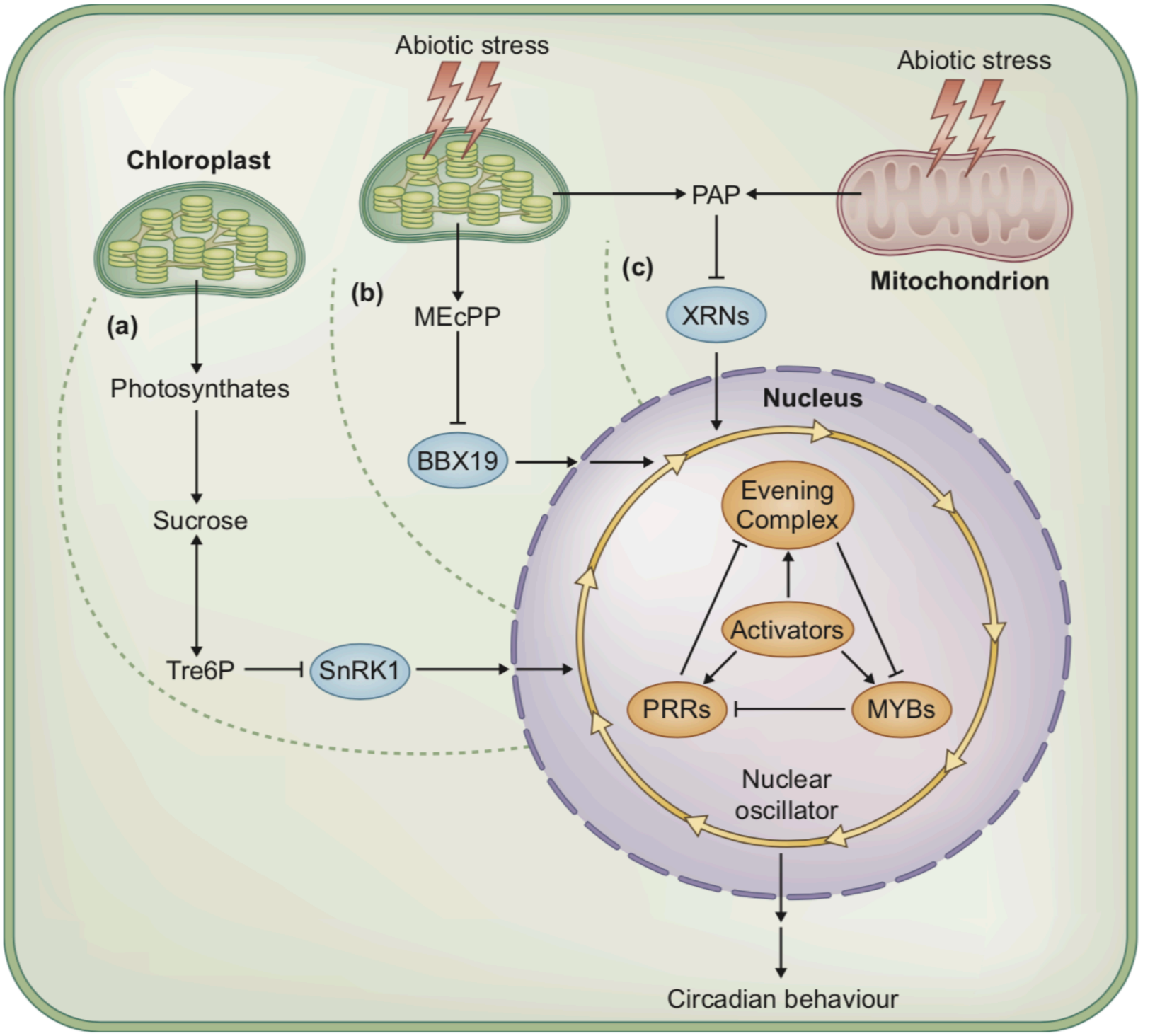
Figure Legends

Figure 1. Operational retrograde signalling contributes to circadian timing in response to environmental factors. The nuclear circadian oscillator consists of multiple negative feedback loops (including Myb transcription factors, PRRs, and the Evening Complex) in complex with activating proteins (summarized in Hsu & Harmer, 2014; Millar, 2016). These transcriptional/translational feedback loops are modified by metabolic intermediaries and products. **(a)** The sucrose/Tre6P nexus regulates the activity of SnRK1 dependent on cytosolic sucrose accumulation. The SnRK1 complex phosphorylates bZIP63, allowing this transcription factor to dimerize and promote the expression of *PRR7*. **(b, c)** The infliction of abiotic stress induces the accumulation of ROS that alters the chloroplast redox state such that MEcPP and PAP accumulate. MEcPP represses the expression of *BBX19*, leading to the accumulation of ELF3 which is a core constituent of the Evening Complex. PAP accumulation inhibits XRN activity, leading to defects in mRNA processing and an extension of circadian period. Abbreviations: ELF3, EARLY FLOWERING3; MEcPP, methylerythritol cyclodiphosphate; PAP, 5'-PhosphoAdenosine 3'-Phosphate; PRRs, PSEUDORESPONSE REGULATORs; ROS, Reactive Oxygen Species; Tre6P, trehalose 6-phosphate; XRN, EXORIBONUCLEASE.


Abiotic stress
Abiotic stress
Chloroplast
PAP
Mitochondrion
(a)
(b)
(c)
MEcPP
XRNs
Photosynthates
Nucleus
BBX19
Evening Complex
Sucrose
Activators
Tre6P
SnRK1
PRRs
MYBs
Nuclear oscillator
Circadian behaviour